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Review

Lysine Biosynthesis in Yeasts*

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S-(β -Aminoethyl)-L-cysteine (SAEC), a sulfur analog of L-lysine, significantly inhibited the growth of wild-type strain of *Candida pelliculosa*. The growth inhibition of *C. pelliculosa* depended on SAEC concentrations, and L-lysine and L- α -amino adipate restored growth. L-Arginine and L-ornithine also recovered the inhibition, though less effectively. SAEC-Resistant mutants were induced from the wild-type strain of *C. pelliculosa* by ultraviolet irradiation and N-methyl-N'-nitro-N-nitrosoguanidine treatment. Almost all the resistant mutants excreted L-lysine into the medium. Lysine excretion increased by repeated mutations. The mutant strain SR-V-1263 extracellularly produced about 2 mg/ml of L-lysine when cultured for 96 hr with shaking. The effect of various factors on lysine production was investigated with the mutant strain. The pH optimum for the production was at 7.0–8.0, and the optimum temperature was 33°C. Glucose was the preferable carbon source, and ammonium sulfate and urea served as good nitrogen sources. The lysine accumulation was markedly stimulated by addition of potassium phosphate, organic acids and organic nitrogen sources. The addition of amino acids and detergents also enhanced the production, though less effectively. The concentration of extracellular lysine reached a maximum (3.2 mg/ml) in the medium containing 2% polypeptone under the experimental conditions used. Lysine biosynthesis and lysine production by yeast mutants also are reviewed with emphasis on the biochemical and enzymological aspects.

KEY WORDS: Yeast mutant/ *Candida pelliculosa*/ Mutation/ Lysine production/ S-(β -Aminoethyl)-L-cysteine (SAEC)/

I. INTRODUCTION

L-Lysine is one of the most important amino acids both nutritionally and biochemically, and is biosynthesized through two distinct pathways, as reviewed by Meister¹⁾ and Broquist and Trupin.²⁾ One of them, α , ϵ -diaminopimelate pathway, occurs in bacteria, certain lower fungi and green plants. The other, α -amino adipate pathway is found in yeasts and molds. In this pathway lysine is synthesized through homocitrate and α -amino adipate from α -ketoglutarate and acetyl-CoA. A number of studies have been devoted to elucidate the biochemical and genetic aspects of the former

* This paper was accepted on the occasion of the Retirement of Prof. Emeritus T. Yamamoto, and was dedicated to him.

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pathway, whereas the α -aminoadipate pathway has not been settled in detail.

L-Lysine is industrially produced by bacterial auxotrophs of glutamate-producing bacteria, e.g., homoserine or threonine auxotrophs of *Corynebacterium glutamicum*³⁾ and *Brevibacterium flavum*.⁴⁾ The resistant mutants to *S*-(β -aminoethyl)-L-cysteine of *B. flavum* also produce lysine effectively.⁵⁾ Only little attention, however, has been paid to lysine production by resistant mutants of yeasts with a few exceptions.

In the present paper, lysine biosynthesis in yeasts is reviewed, and lysine production by yeast mutants resistant to some lysine analogs, particularly by *S*-(β -aminoethyl)-L-cysteine resistant mutants of *Candida pelliculosa* also is described.

II. α -AMINOADIPATE PATHWAY

II-1. Studies on the α -Aminoadipate Pathway

The α -aminoadipate pathway has been studied mainly with *Saccharomyces cerevisiae* and *Neurospora crassa*. Several lysine auxotrophic mutants of *N. crassa* were isolated, and one of them was capable utilizing α -aminoadipate in place of lysine.⁶⁾ [¹⁴C]- α -Aminoadipate in a medium was effectively incorporated into lysine by a fungal mutant, but not into other amino acids.⁷⁾ These findings suggest that α -aminoadipate is a precursor of lysine in *N. crassa*. Certain lysine auxotrophs of the yeast, *Ophiostoma multiannulatum* grew in a medium supplemented with α -aminoadipate,⁸⁾ showing that lysine is also synthesized through this amino acid in yeasts. A few *N. crassa* mutants could grow on a minimum medium supplemented with α -amino- ϵ -hydroxycaproate.⁹⁾

Strassman and Weinhouse¹⁰⁾ proposed, based on isotopic labelling experiments with a yeast, a possible mechanism for formation of the carbon skeleton of lysine: the methyl carbon atom of acetate may condense with the carbonyl carbon atom of α -ketoglutarate to yield a seven-carbon homolog of citrate (homocitrate) followed by a sequence of reactions homologous to the formation of α -ketoglutarate *via* reactions of the citric acid cycle. Studies with yeast extracts supported this mechanism: the enzymatic formation of labelled homocitrate¹¹⁾ and radioactive α -ketoadipate from [¹⁴C]-acetate and α -ketoglutarate,¹²⁾ the conversion of homoisocitrate into α -ketoadipate^{13,14)} and the formation of *cis*-homoaconitate from homoisocitrate¹⁵⁾ were demonstrated. An extract from a lysine auxotroph of *N. crassa* was also reported to catalyze the formation of homocitrate from acetate and α -ketoglutarate.¹⁶⁾ In addition, the accumulation of homocitrate,¹⁶⁻²⁰⁾ *cis*-homoaconitate^{15,17,18,21)} and homoisocitrate^{15,18)} in growth medium by certain lysine requiring mutants of *S. cerevisiae* and *N. crassa* also was shown. α -Aminoadipate was formed by transamination from α -ketoglutarate. Betterton *et al.*²²⁾ reported that yeast extract catalyzes transamination between glutamate and α -ketoadipate to yield α -aminoadipate. Data showed the participation of α -aminoadipate as an intermediate in lysine biosynthesis, and many investigations were devoted to elucidate the metabolic fate of α -aminoadipate.

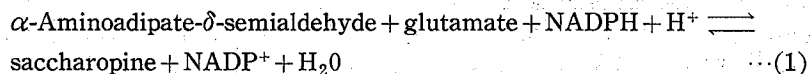
Sagisaka and Shimura^{23,24)} reported that yeast extract catalyzes the conversion of α -aminoadipate- δ -semialdehyde in the presence of ATP, NADH, Mg²⁺ and reduced

glutathione, and also catalyzes an aminoadipate-dependent ATP-pyrophosphate exchange. Larson *et al.*²⁵⁾ also observed the enzymatic conversion of α -aminoadipate into the corresponding δ -semialdehyde. They observed that an orange compound was formed, when yeast resting cells were incubated with α -aminoadipate in the presence of *o*-aminobenzaldehyde. It was postulated that α -aminoadipate- δ -semialdehyde, which also exists in a cyclized form as Δ' -piperideine-6-carboxylate, is formed by reduction of α -aminoadipate, and that *o*-aminobenzaldehyde reacts with the piperideine carboxylate to form a dihydroquinazolium compound.²⁵⁾ These suggest that the conversion of α -aminoadipate into the aldehyde is a two-step process: α -aminoadipate reacts with ATP to form α -aminoadipic- δ -adenylate followed by reduction to the corresponding aldehyde in the presence of NADH. The α -aminoadipic- δ -adenylate was isolated by chromatography after incubation of yeast extract with α -aminoadipate, ATP and Mg^{2+} ,²⁶⁾ and was also isolated by culture broth in which a lysine-less mutant of *S. cerevisiae* was grown.²⁷⁾

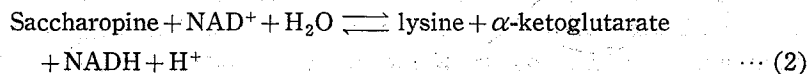
In *N. crassa* mutants that could utilize α -amino- ϵ -hydroxycaproate, a cell-free preparation was found to catalyze the reversible oxidation of α -amino- ϵ -hydroxycaproate to α -aminoadipate- δ -semialdehyde.²⁸⁻³⁰⁾ Thus, it is likely that the ϵ -hydroxy compound utilization involves the conversion to the aldehyde followed by oxidation to α -aminoadipate.

Kue *et al.*³¹⁾ found that incubation of [^{14}C]- α -aminoadipate with resting cells of *S. cerevisiae* led to formation of an unknown radioactive product, "Compound B", which was converted to labelled lysine by further incubation. The "Compound B" was purified and identified as saccharopine [ϵ -*N*-(L-glutaryl-2)-L-lysine],³¹⁾ which was first discovered by Kjaer and Larsen.³²⁾ Saccharopine serves as an intermediate in lysine biosynthesis. Studies with cell-free extracts of yeast mutants showed that radioactive saccharopine was formed from [^{14}C]- α -aminoadipate- δ -semialdehyde in the presence of NADPH and glutamate.³³⁾ A lysine-requiring mutant of *N. crassa* accumulates labelled saccharopine, when grown in [^{14}C]- α -aminoadipate medium.³⁴⁾

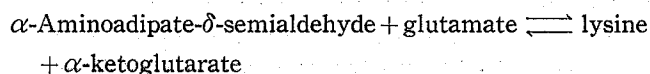
The enzyme, α -aminoadipate semialdehyde-glutamate reductase, which was first named as saccharopine reductase by Jones and Broquist,³³⁾ was purified approximately 130-fold from *S. cerevisiae*.³⁵⁾ The enzyme catalyzes the following reversible reaction:



Saccharopine dehydrogenase catalyzes the conversion of saccharopine to lysine (the equation 2) and was purified about 700-fold from baker's yeast.³⁶⁾



Coupling both enzymes results in a transamination:

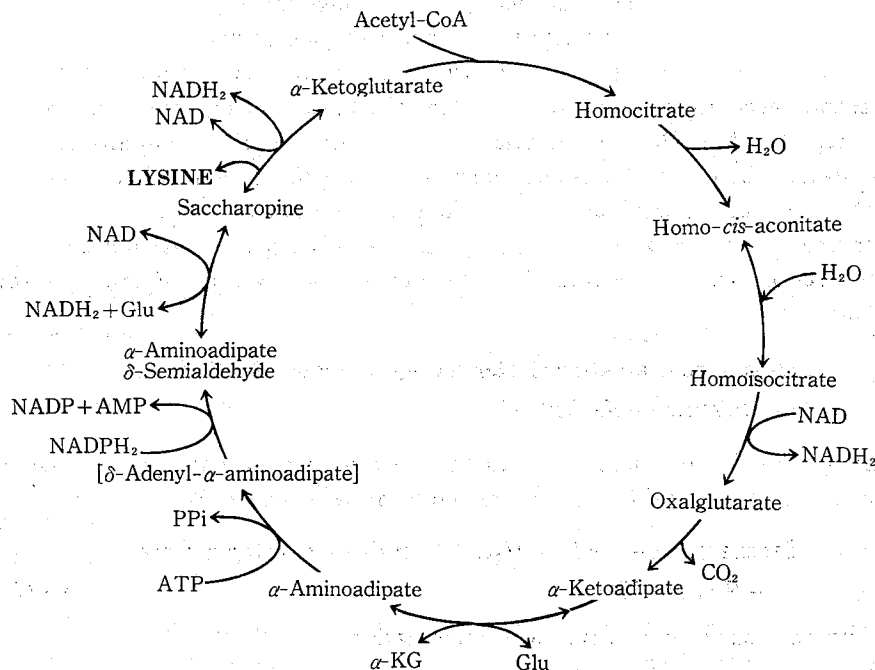


In this reaction saccharopine serves as a stable intermediate, and pyridoxal phosphate is not required as a coenzyme, but NADP is. Recently, saccharopine dehydrogenase from *S. cerevisiae* was purified to homogeneity, and its enzymological properties were investigated.

It was shown that certain lysine-less mutants of *Rhodotorula glutinis* blocked between α -aminoadipate and lysine can utilize L-pipecolate, although this compound fails to support growth of several lysine auxotrophs of *S. cerevisiae*,³⁷⁾ and also that extracts of wild-type *R. glutinis* catalyze the conversion of L-pipecolate to α -aminoadipate- δ -semialdehyde.³⁷⁾ Thus, L-pipecolate is utilized for biosynthesis of L-lysine in *R. glutinis*, but not in *S. cerevisiae*. The biosynthetic role for L-pipecolate is also found in the lysine pathway of *Aspergillus nidulans*³⁸⁾ and *Euglena gracilis*,³⁹⁾ though the detailed mechanism remains unsettled.

Certain auxotrophic mutants of *S. cerevisiae* have been reported to accumulate glutarate.⁴⁰⁾ A cell-free preparation of the wild-type yeast was found to decarboxylate α -ketoacidipate to yield glutaric- γ -semialdehyde.⁴¹⁾ It is likely that the glutarate accumulation is caused by decarboxylation of α -ketoacidipate accumulated due to a block in the pathway, but glutarate is not an intermediate in lysine biosynthesis.⁴¹⁾

A proposed mechanism of lysine biosynthesis in yeasts and molds is shown in Scheme 1.

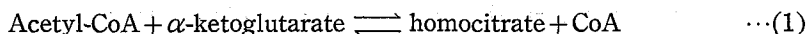


Scheme 1. Lysine biosynthesis in yeasts and molds.
(α -Aminoacidipate pathway)

II-2. Enzymes of the α -Aminoadipate Pathway

The initial steps of lysine biosynthesis *via* the α -aminoadipate pathway are analogous with the condensation of acetyl-CoA and oxalacetate to form citrate and the subsequent conversion of citrate into isocitrate and α -ketoglutarate.¹⁰⁾ Betterton *et al.*²²⁾ showed that in a yeast the enzymes participating in the initial steps of the pathway leading to α -aminoadipate are localized in the mitochondria as the enzymes of TCA cycle are. Although evidence for each step in the pathway has been obtained as described above, the enzymological studies have not been performed in detail, except saccharopine dehydrogenase.

(1) Homocitrate synthase



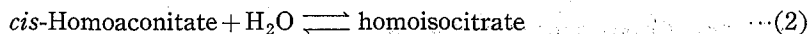
Homocitrate synthase catalyzes the first step of lysine biosynthesis: the condensation of α -ketoglutarate and acetyl-CoA. The enzyme activity has been demonstrated in cell-free extracts of *S. cerevisiae*,^{11, 17, 42-44)} *N. crassa*¹⁶⁾ and *Penicillium chrysogenum*.^{45, 46)} Radioactive homocitrate was isolated and analyzed by chromatography after incubation with [¹⁴C]-acetate, acetyl-CoA generating system and α -ketoglutarate. The acetyl-CoA generating system is composed of ATP, CoA, Mg²⁺ and acetyl-CoA synthase of the extracts. Although purification of the enzyme has not been extensively achieved, Tucci and Ceci⁴³⁾ have observed two peaks of the enzyme activity by isoelectric focusing chromatography with the extract of *S. cerevisiae*. No evidence for the occurrence of isozymes was obtained in *Saccharomycopsis lipolytica*.⁴⁷⁾

Gaillardin *et al.*⁴⁷⁾ studied yeast homocitrate synthase kinetically. The fixation of one substrate, acetyl-CoA shows sigmoidal saturation kinetics, and the enzyme reaction proceeds through an ordered mechanism, in which α -ketoglutarate binds to the enzyme followed by acetyl-CoA.⁴⁷⁾

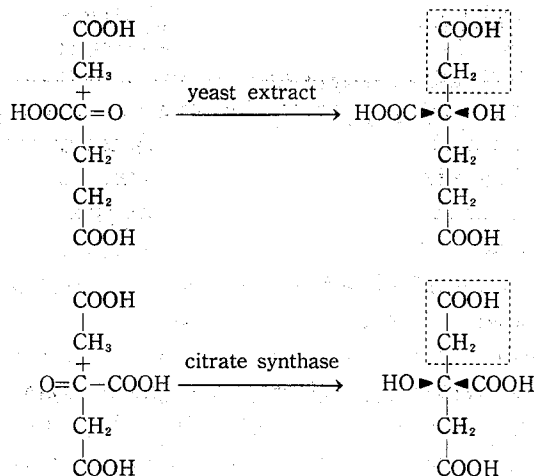
Thomas *et al.*⁴⁸⁾ investigated the absolute configuration of homocitrate formed enzymatically. They synthesized (*S*)-2-hydroxy-1, 2, 4-butanetricarboxylic acid lactone and compared this isomer with the homocitrate preparation isolated by Maragoudakis and Strassman¹⁷⁾ from a yeast lysine-less mutant. They showed that the homocitrate is (*R*)-2-hydroxy-1, 2, 4-butanetricarboxylic acid.⁴⁸⁾ Acetate moiety is added to the carbonyl carbon of α -ketoglutarate in a position opposite to that found in synthesis of citrate by citrate synthase (Scheme 2).⁴⁸⁾

Homocitrate synthase is feedback-controlled by lysine, as described below.

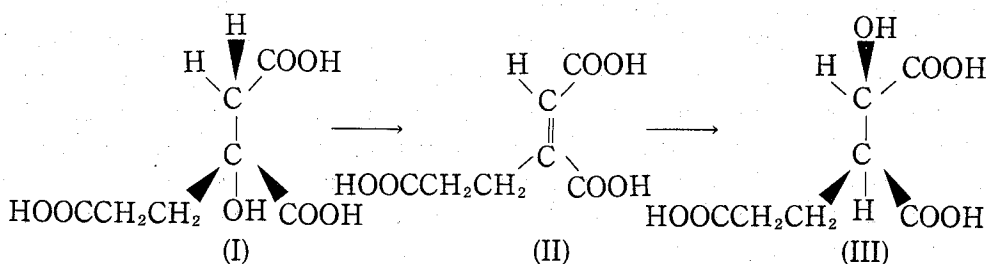
(2) *cis*-Homoaconitase



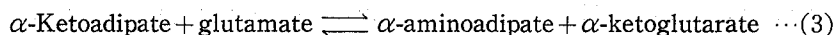
cis-Homoaconitase catalyzes the reversible conversion of *cis*-homoaconitate to homoisocitrate, and was partially purified from baker's yeast.¹⁵⁾ It is different from aconitase from the same source in the following respects: 1) homoaconitase can be differentiated by ammonium sulfate fractionation; *cis*-homoaconitase activity is found in the fraction between 40–50% of the saturation, but aconitase activity is not; 2) the pH optimum of homoaconitase and aconitase are 8.5 and 8.7, respectively; 3) The effect of various inhibitors are different in both enzymes.

Scheme 2. Stereochemistry of enzymatic formation of homocitrate and citrate.⁴⁸⁾

Chilina *et al.*⁴⁹⁾ showed that homoisocitrate enzymatically formed has the absolute configuration of *threo*-D₂-homoisocitrate [1-(*R*)-hydroxy-2(*S*)-1, 2, 4-butanetricarboxylic acid]. The stereochemistry of the enzymatic conversion of homocitrate to homoisocitrate is analogous to that of citrate to isocitrate: the rearrangement seems to occur by *trans*-elimination and *trans*-addition of the elements of water with accompanying by a reversal of the position of attachment of the hydrogen and hydroxyl groups (Scheme 3).⁴⁹⁾

Scheme 3. Stereochemistry of the conversion of homocitrate (I) into homoisocitrate (III).⁴⁹⁾

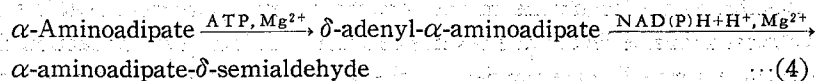
(3) α -Aminoadipate aminotransferase



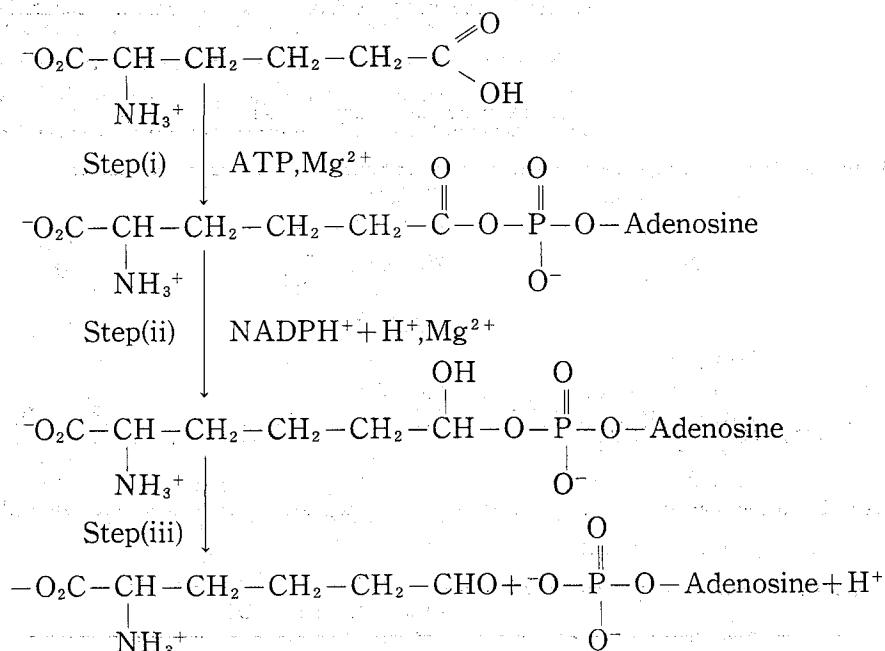
Two forms of the aminotransferase were demonstrated in a yeast extract by DEAE-cellulose column chromatography: one is in mitochondria and the other is in cytoplasm.²²⁾ Molecular weights of the mitochondrial and cytoplasmic enzymes were determined to be approximately 100,000 and 140,000, respectively by Sephadex G-200 gel filtration.²²⁾

α -Ketoadipate added in medium is efficiently converted into lysine by yeast cells.^{50,51} This suggests that the aminotransferase participates in lysine biosynthesis.

(4) α -Aminoadipate reductase system

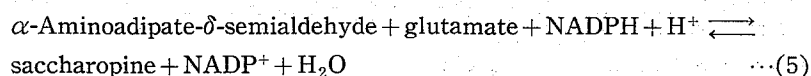


The reduction of α -aminoadipate to α -aminoadipate- δ -semialdehyde has been shown with yeast extracts as described above. Sagisaka and Shimura²³⁾ based on studies with yeast extracts postulated that the reduction may proceed *via* two steps including the postulated intermediates as described above. Sinha and Bhattacharjee^{26, 52)} later demonstrated that the conversion consists of three steps (Scheme 4). α -Amino adipate is activated by ATP to form δ -adenyl- α -amino adipate, which is subsequently reduced in the presence of NADPH. The reduced adenylyl derivative of the α -amino adipate is cleaved to yield α -amino adipate- δ -semialdehyde. The step (iii) is also the enzyme reaction, because two lysine auxotrophs of *S. cerevisiae* (ly₂ and ly₅) lacking the step (iii) have been obtained. δ -Adenylyl- α -amino adipate was isolated by chromatography after incubating a cell-free extract of *S. cerevisiae* with α -amino adipate, ATP and Mg²⁺, but the reduced δ -adenylylsemialdehyde was too labile to be identified.²⁶⁾



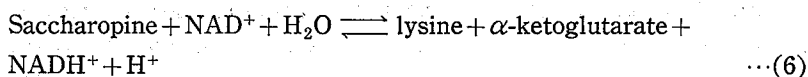
Scheme 4. Proposed mechanism for the reduction of α -amino adipate to α -amino adipate- δ -semialdehyde.³⁶⁾

(5) α -Aminoadipate semialdehyde-glutamate reductase



The enzyme catalyzes the reversible reaction (5), and was purified approximately 130-fold from baker's yeast.³⁵⁾ The pH optimum is in the range of 7 for the forward reaction, and it is at about pH 10 for the reverse reaction. NADPH is more active than NADH as the coenzyme in the forward reaction, whereas NADP⁺ and NAD⁺ show almost similar effect in the reverse reaction. In the reverse reaction the amount of glutamate formed corresponded to the amount of NADP⁺ reduced, though the stoichiometry of the forward reaction has not been settled. The Michaelis constants for saccharopine and NADP⁺ are 0.92 and 0.022 mM, respectively. The enzyme from baker's yeast has an $S_{20,w}$ value of 4.9 and a molecular weight of approximately 73,000, which was estimated by sucrose density centrifugation. The enzyme was completely inhibited by 1 mM pCMB, suggesting that the enzyme is a sulfhydryl enzyme.

(6) Saccharopine dehydrogenase



Saccharopine dehydrogenase was purified by more than 700-fold from *S. cerevisiae*.³⁶⁾ Recently, Ogawa and Fujioka⁵³⁾ purified the enzyme to homogeneity from baker's yeast by a procedure including 5'-AMP-Sepharose column chromatography. Enzymological properties of the enzyme from baker's yeast are summarized in Table I.

Table I. Properties of Saccharopine Dehydrogenase (*Saccharomyces cerevisiae*)

Molecular weight	40,300* ^{a)} 38,000 ± 3,000* ^{b)} 39,000 ± 2,000* ^{c)} 37,000 ± 5,000* ^{d)} 49,000** ^{e)}
Sedimentation coefficient ($S_{20,w}$)*	3.0 ± 0.3S
NH ₂ -terminal amino acid*	Alanine
Isoelectric point*	10.1
pH Optima**	pH 6.0–6.5 (Reverse reaction) pH 10 (Forward reaction)
Michaelis constants**	1.2 × 10 ⁻² M (for L-Lysine) 4.4 × 10 ⁻⁴ M (for α-Ketoglutarate) 4.6 × 10 ⁻⁵ M (for NADH)

* From ref. (53).

** From ref. (36).

a) From Sephadex gel filtration.

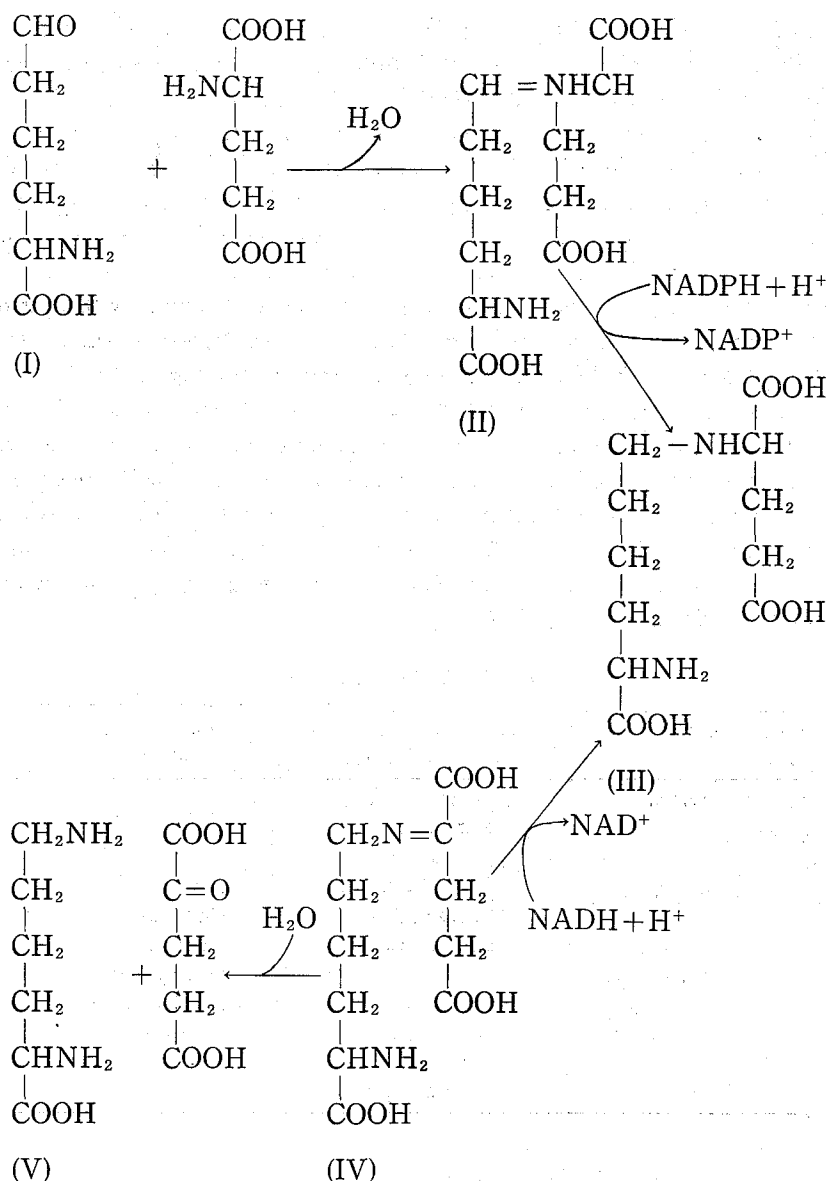
b) From sedimentation equilibrium.

c) From dodecyl sulfate-polyacrylamide gel electrophoresis.

d) From Svedberg equation.

e) From Sucrose density gradient.

Saccharopine dehydrogenase is coupled with aminoadipate semialdehyde-glutamate reductase to catalyze an overall transamination of α -aminoadipate semialdehyde to lysine *via* a stable intermediate, saccharopine. Saunders and Broquist³⁶⁾ presented a possible mechanism of the transamination of α -aminoadipate- δ -semialdehyde with glutamate to yield lysine (Scheme 5). They postulated the Schiff bases (II) and (IV) in the course of the reaction. α -Aminoadipate- δ -semialdehyde



Scheme 5. Proposed mechanism of transamination of α -aminoadipate- δ -semialdehyde by glutamate to yield lysine.³⁶⁾

may be condensed with glutamate to yield an intermediate (II) followed by reduction to a stable intermediate, saccharopine (III). Saccharopine is oxidized by NAD^+ to give the Schiff base (IV), which is hydrolyzed to form lysine and α -ketoglutarate.

Fujioka and Nakatani⁶⁴) kinetically studied saccharopine dehydrogenase. Initial velocity and product-inhibition studies showed that the enzyme reaction proceeds through an ordered mechanism: the sequence of binding of substrate to the enzyme is NAD^+ and saccharopine, and that of release of products is lysine, α -ketoglutarate and NADH .

Lysine is degraded through saccharopine and α -aminoadipate as intermediates in mammalian tissues:⁵⁵⁻⁶⁰) the catabolic pathway (saccharopine pathway) is in reverse order of the lysine biosynthetic pathway. A mammalian enzyme, L-lysine- α -ketoglutarate reductase (saccharopine-forming) catalyzes the same reaction as saccharopine dehydrogenase.^{56, 61, 62}) The reductase, however, is different from the yeast saccharopine dehydrogenase in several respects: 1) coenzyme specificity; the former requires NADP^+ as a coenzyme, but the latter does not; 2) molecular weight; the former is an oligomeric enzyme composed of four subunits with molecular weight of approximately 50,000, whereas the latter is a monomeric enzyme with that of about 39,000; 3) the former catalyzes only the reaction in the direction of oxidative cleavage of saccharopine, whereas the latter catalyzes the reversible reaction.

Saccharopine dehydrogenase has been found in certain lower fungi, higher ascomycetes and euglenids, although diaminopimelate decarboxylase, which catalyzes the terminal step of the diaminopimelate pathway, has not been found in these organisms. Vogel⁶³) suggested that this enzyme is a marker enzyme for the α -aminoadipate pathway of lysine biosynthesis in plants. He extensively investigated the distribution of the two lysine pathways in various organism and discussed the possible evolutionary significance (Table II).

Table II. Lysine Biosynthesis⁶³)

	α , ϵ -Diaminopimelate pathway	α -Aminoadipate pathway
<i>Bacteria</i>	Pseudomonas Eubacteria Actinomycetes	
<i>Lower fungi</i>	Hyphochytriales Saprolegniales Leptomitales	Chytrids Blastocladales Mucorales
<i>Higher fungi</i>		Ascomycetes Basidiomycetes
<i>Green organisms</i>	Green algae Ferns Flowering plants	Euglenids

III. CONTROL OF LYSINE IN THE α -AMINOADIPATE PATHWAY

Lysine biosynthesis in yeasts and molds is feedback-controlled by lysine.

Maragoudakis *et al.*¹⁹⁾ and Tucci⁴²⁾ reported that the formation of radioactive homocitrate by intact cells and cell-free extracts of *Saccharomyces cerevisiae* decreases in the presence of lysine. Hogg and Broquist¹⁶⁾ also showed that lysine inhibits homocitrate synthase of *Neurospora crassa*. Thus, homocitrate synthase, the first enzyme of the pathway is a control site. Two forms of homocitrate synthase are found in *S. cerevisiae*, and both are equally inhibited by lysine, and one is repressed to a greater extent by excess lysine in medium.⁴³⁾ The enzyme from *N. crassa* is also repressed by lysine.⁴⁶⁾

Tucci and Ceci,⁴⁴⁾ however, observed that the incorporation of radioactivity of [¹⁴C]- α -aminoadipate into lysine is markedly inhibited by exogenous lysine in *S. cerevisiae*, and homocitrate synthase *in vitro* is much less sensitive to lysine inhibition. Therefore, they suggested that homocitrate synthase plays only a minor role in the regulation of the pathway, and the control site of lysine exists between α -aminoadipate and lysine. A control site at the middle of a pathway usually occurs when a metabolic pathway branches. For example, a branch in the pathway leading to the biosynthesis of penicillin is known in *Penicillium chrysogenum*.⁶⁴⁾ They, however, could not obtain evidence for the occurrence of a branch in lysine biosynthesis in the yeast.⁴⁴⁾

Biosyntheses of lysine and penicillin are linked in *P. chrysogenum* since α -aminoadipate is a common intermediate. It has been reported that lysine inhibits penicillin biosynthesis in *P. chrysogenum*,⁶⁵⁾ and that α -aminoadipate can restore the inhibition of penicillin formation by lysine.⁶⁶⁾ Masurekar and Demain⁶⁷⁾ suggested that the lysine inhibition was due to a negative feedback control of an initial enzyme of the common pathway. They found that the accumulation of labelled homocitrate by a mutant of *P. chrysogenum* is inhibited by exogenous lysine.⁶⁸⁾ They later demonstrated that homocitrate synthase is markedly inhibited by both lysine and benzylpenicillin, a precursor of penicillin, though the enzyme is insensitive to lysine inhibition.⁶⁹⁾ This suggests that the enzyme is regulated by a concerted feedback inhibition by both products.

IV. CONTROL OF LYSINE BIOSYNTHESIS BY LYSINE ANALOGS

Certain lysine analogs have been reported to inhibit the first enzyme in yeasts. Sinha *et al.*⁷⁰⁾ investigated the effect of δ -hydroxylysine on lysine biosynthesis in *S. cerevisiae*, and found that the analog inhibits homocitrate synthesis and also decreases the level of α -aminoadipate semialdehyde-glutamate reductase. This analog also inhibits the growth of *Streptococcus faecalis* by limiting the cellular source of lysine, and inhibits the first enzyme of diaminopimelate pathway, aspartokinase.⁷¹⁾ Gaillardin *et al.*⁴⁷⁾ reported that δ -hydroxylysine, *trans*-4, 5-dehydrolysine and 3-aminocyclohexane alanine markedly inhibits homocitrate synthase activity in cell-free extract of *Saccharomyces lipolytica*, though *S*-(β -aminoethyl)-L-cysteine (SAEC) does not. These analogs act as true lysine analogs, binding to the same site(s) of lysine on the enzyme: they show cooperative binding and have an heterotropic effect on α -ketoglutarate binding sites.⁴⁷⁾

SAEC strongly inhibits aspartokinase of *Escherichia coli* as a false feedback in-

hibitor,⁷²⁾ and also homocitrate synthase of *S. cerevisiae*.⁷³⁾ Recently, we found that this sulfur analog markedly inhibits homocitrate synthase of *Candida pelliculosa*,⁷⁴⁾ and the action of the analog on the enzyme is currently under investigation.

V. LYSINE PRODUCTION BY LYSINE SULFUR ANALOG-RESISTANT MUTANTS OF *CANDIDA PELLICULOSA*

Since the studies by Adelberg and Cohen,^{75,76)} amino acid analogs and microbial mutants resistant to them have been used to investigate amino acid metabolism and its regulatory mechanisms.⁷⁷⁾ Some of the mutants have been utilized to produce amino acids industrially as well.⁷⁸⁻⁸⁰⁾ For example, the resistant mutants to *S*-(β -aminoethyl)-L-cysteine (SAEC) of *Brevibacterium flavum* produce lysine effectively.⁵⁾

Lysine production by yeasts was investigated with several analog-resistant mutants of *Saccharomyces cerevisiae*^{81,82)} and *Saccharomycopsis lipolytica*.⁸³⁾ *S. cerevisiae* has an advantage for lysine production since the biochemical and genetic aspects of its lysine biosynthesis have been studied.⁸²⁾ Haidaris and Bhattacharjee^{81,82)} have induced many mutants resistant to SAEC from *S. cerevisiae*. The biochemical mechanism of the lysine production by the mutants has not been elucidated. Gaillardin *et al.*⁸³⁾ also have obtained the mutants resistant to *trans*-4, 5-dehydrolysine at a high frequency by ultraviolet irradiation in both haploid and diploid strains of *Saccharomycopsis lipolytica*. The regulatory mutant, strain *mg-5 lex*⁺, in which homocitrate synthase is desensitized, can accumulate lysine intracellularly, but not extracellularly. Thus, they induced several lysine-excreting mutants (*mg-5 lex*⁻) by repeated UV irradiation. These strains excreted lysine in a medium at the end of the exponential phase with a concomitant decrease in intracellular lysine. This excretion is ascribable to neither a release of intracellular lysine nor to autolysis of the cells. Although *Candida* yeast has not been used in food industry, it has some advantage over *S. cerevisiae* for potential lysine production. First, the cells of some *Candida* strains *e.g.*, *C. pelliculosa* and *C. guilliermondii* var. *membranaefaciens* have been reported to be in a haploid form.^{84,85)} Tsukada and Sugimori⁸⁶⁾ have induced auxotrophic mutants from *C. pelliculosa* by UV irradiation with a high mutation rate. Secondly, the *Candida* yeast seems capable of excreting metabolites into medium.⁸⁷⁾

We have isolated SAEC-resistant mutants of *C. pelliculosa* excreting lysine effectively.^{88,89)} In this section, the effect of SAEC on the growth of *C. pelliculosa* and induction of SAEC-resistant mutants of *C. pelliculosa* are described. The effect of cultural conditions on lysine production by the mutant strains is also described.

(1) Effect of SAEC on Growth of Wild-Type Strain of *Candida pelliculosa*

We examined whether SAEC acts as a metabolic antagonist against L-lysine. *C. pelliculosa* (IFO 0707) was used throughout the experiments. SAEC was synthesized from L-cysteine and ethyleneimine by a modification⁹⁰⁾ of the method of Cavallini *et al.*⁹¹⁾ The minimum and complete media were prepared according to the procedure of Lingens and Oltmanns⁹²⁾. To examine the effect of SAEC on growth of *C. pelliculosa*, 0.1 ml of a seed culture was transferred into 4 ml of the minimum medium containing various concentrations of SAEC in a test tube and incubated at 28°C on a

reciprocating shaker. Cell growth was measured turbidimetrically at 660 nm, with a Hitachi photoelectric photometer (SPQ-B).

The growth of the wild-type strain of *C. pelliculosa* was significantly inhibited by SAEC (Fig. 1). The initiation of the growth was delayed with increasing concentrations of SAEC: *C. pelliculosa* could not grow within about 70 hr when 0.2% SAEC was added. The organisms, however, grew at a normal rate after a lag period. We observed that the wild-type strain did not mutate spontaneously during prolonged cultivation under the conditions used.

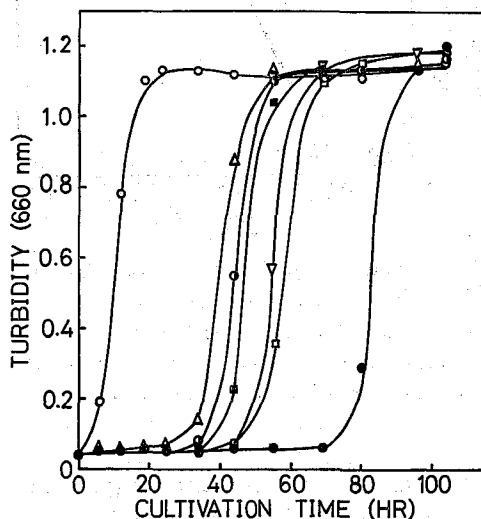


Fig. 1. Effect of SAEC on growth of wild-type strain of *Candida pelliculosa*.

A 0.1-ml aliquot of the seed suspension was inoculated into the Lingens-Oltmanns' minimum medium supplemented with SAEC.

○, None; △, 0.01%; ●, 0.025%; ■, 0.05%; △, 0.075%; □, 0.1%; ●, 0.2%.

We have found that *Aerobacter aerogenes* is capable of utilizing SAEC as a sole nitrogen source,⁹³⁾ and that SAEC is metabolized to *S*-(β -*N*-acetyl aminoethyl)-L-cysteine by acetyl-CoA: SAEC ω -*N*-acetyltransferase of the organism.^{94,95)} Guengerich and Broquist⁹⁶⁾ showed lysine metabolism *via* ϵ -*N*-acetyllysine in *Rhizoctonia leguminicola*, although they did not study the acetylation of the sulfur analog. Several other enzymes catalyzing decomposition of SAEC have been demonstrated, *e.g.*, L-lysine decarboxylase of *Bacterium cadaveris*,⁹⁷⁾ and L-lysine- α -ketoglutarate ϵ -amino-transferase of *Achromobacter liquidum*.⁹⁸⁾ Thus, it is likely that one of these enzymes is induced or activated by SAEC during growth of *C. pelliculosa* to detoxicate it.

(2) Reversal of SAEC-Inhibition

The effect of L- and D-lysine or related compounds was examined to elucidate whether inhibition by SAEC was related to lysine metabolism. The addition of L-lysine or L- α -aminoadipate, a precursor of lysine, effectively eliminated growth inhibition by SAEC in proportion to the concentrations, though D-lysine was inert (Fig. 2). The protective effect of L- α -aminoadipate was higher than that of L-lysine:

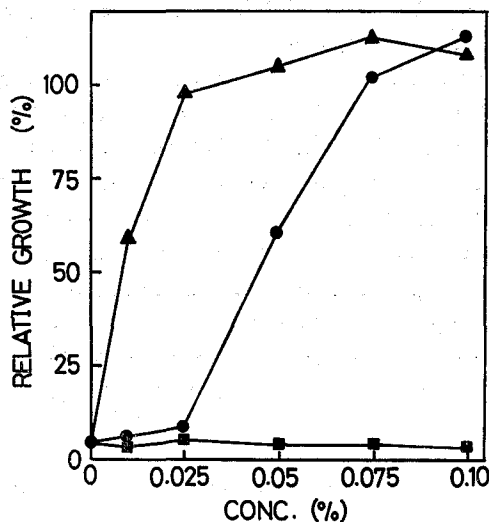


Fig. 2. Reversal of SAEC-inhibition by L-lysine (●), D-lysine (■) and L-α-aminoadipate (▲).

A 0.1-ml aliquot of the seed suspension was inoculated into the Lingens-Oltmanns' minimum medium containing 0.5% SAEC and various concentrations of L- and D-lysine and L-α-aminoadipate. The turbidity was measured after 49 hr inoculation when the control culture showed maximum turbidity.

L-lysine and L-α-aminoadipate at concentrations higher than about 0.075% and 0.025%, respectively, restored almost fully the growth inhibition in the presence of 0.5% SAEC. It is likely that L-α-aminoadipate is transported into cells more rapidly than L-lysine, and converted effectively to L-lysine.

L-Ornithine, L-arginine and L-glutamate also recovered inhibition, although less effectively. The effect of L-ornithine and L-arginine can probably be ascribed to their structural similarity to L-lysine. Other L- and D-amino acids tested, homocitrate, α-ketoglutarate, nucleotides and vitamins were not at all or only slightly effective. The specific recovery of SAEC-inhibition by L-lysine and L-α-aminoadipate suggests that SAEC interfered directly with lysine biosynthesis. Therefore, a regulatory mutant which is resistant to SAEC, a potent antimetabolite of L-lysine, is probably suitable for lysine overproduction.

(3) Induction and Screening of SAEC-Resistant Mutants

Attempts were made to induce SAEC-resistant mutants from the wild-type strain of *C. pelliculosa*. The cells grown on the complete medium at 28°C for 24 hr were harvested, washed twice with the Lingens-Oltmanns' washing solution,⁹²⁾ and suspended into the solution. The cell suspension was irradiated with ultraviolet light (UV) or treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as follows.

1) UV irradiation. The cell suspension (10 ml, $0.8-1 \times 10^8$ cells/ml) placed in a Petri dish of 8.5 cm diameter were exposed to UV (National GL-15 15 W germicidal lamp) for 3 min at a distance of 40 cm.

2) NTG treatment. The same suspension was treated with 0.2% NTG in 0.2 M

sodium phosphate buffer (pH 6.0) at 28°C for 20 min and then washed twice with the washing solution to remove the mutagen.

The cells treated with UV or NTG were spread on the complete agar medium and incubated at 28°C for 72 hr. Colonies were transferred to the minimum agar medium supplemented with 0.3% SAEC. Colonies appearing on the plate within 72 hr were picked up as SAEC-resistant mutants. The mutants obtained grew well even in the presence of 0.5% SAEC.

UV irradiation (3 min) giving the survival rate of 0.1% was almost effective for the mutation, and the rate optimum for the NTG treatment was $6 \times 10^{-6}\%$.

The relationship between the frequency of the appearance of the resistant colonies and concentrations of SAEC supplemented in the screening agar plate was investigated. The high frequency was observed at the low concentrations of less than 0.3% of SAEC (Table III).

Table III. Induction of SAEC-Resistant Mutants from The Wild-type Strain of *Candida pelliculosa*

The cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were suspended in water and spread on agar plates containing various concentrations of SAEC. Colonies appeared were counted after incubation at 30°C for 72 hr.

Conc. of SAEC in the medium (%)	Surviving cells ^{a)} (Cells/ml) (Control)	Resistant-colonies counted (Cells/ml)	Frequency (%)
0.1	9,000	32	0.360
0.2	5,200	18	0.350
0.3	1,600	0.55	0.034
0.4	700	0.25	0.036
0.5	350	0.12	0.034

a) Surviving cells in the suspension treated with NTG.

SAEC-Resistant mutants (951 strains) induced by the first mutation (NTG treatment) were cultured to examine the productivity of lysine with screening medium A. The medium A was composed of 10% glucose, 2% $(\text{NH}_4)_2\text{SO}_4$, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2% KH_2PO_4 , 1% vitamin solution⁹²⁾ and 0.5% trace element solution.⁹²⁾ L-Lysine accumulated in the medium was determined spectrophotometrically by the acid ninhydrin method.⁹⁹⁾ The wild-type strain produced no appreciable amount of lysine extracellularly, but almost all the resistant mutants did. When the screening agar plates containing a high concentration of SAEC was used, no good excretors were induced (Table IV). No clear relationship was found between lysine excreting ability of the mutants and their resistibility to SAEC. Strain SR-I-69 excreting 0.6–0.8 mg/ml of lysine in screening medium B was selected among SAEC-resistant mutants induced with medium containing 0.3% SAEC. The screening medium B was composed of 10% glucose, 2% urea, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.8% KH_2PO_4 , 1% vitamin solution⁹²⁾ and trace element solution.⁹²⁾ Because a high concentration of KH_2PO_4 enhanced lysine productivity as described

Table IV. Lysine Productivity of SAEC-Resistnat Mutants Induced by the First Mutation in the Presence of Various Concentrations of SAEC

Conc. of SAEC in the medium (%)	Total number of the mutants ^{a)}	L-Lysine produced (mg/ml) and Number of lysine-producing mutants ^{b)}		
		<0.1	0.1-0.2	>0.2
0.1	124	32 (25.8%)	89 (71.8%)	3 (2.4%)
0.2	133	82 (61.7%)	50 (37.6%)	1 (0.7%)
0.3	257	90 (35.0%)	165 (64.2%)	2 (0.8%)
0.4	204	116 (56.7%)	88 (43.2%)	0
0.5	233	222 (94.9%)	12 (5.1%)	0

a) The resistant mutants were obtained by the first mutation with the NTG treatment.

b) The mutants were cultured in the screening medium A in a test tube at 28°C for 96 hr. L-Lysine was determined by the acid ninhydrin method.

below, 0.8% KH_2PO_4 was involved in the medium.

The lysine productivity of SR-I-69 mutant was increased by further mutations. Distribution patterns of lysine productivity by the mutants were presented in the accompanying paper.⁷⁴⁾ Among 195 resistant mutants obtained by five-fold mutations, about 85% excreted several other ninhydrin-positive compounds than lysine. The compounds were identified as ornithine, glutamate and proline by paper chromatography. The most potent lysine producer, SR-V-1263 mutant, which excreted 2.2 mg/ml of lysine in the screening medium B, was used throughout the following experiments.

(4) Time course of Lysine Production by Strain SR-V-1263

SR-V-1263 mutant was grown to accumulate lysine at 28°C for 96 hr with 4 ml of the basal fermentation medium in a test tube with reciprocal shaking. The basal fermentation medium contained 10% glucose, 2% $(\text{NH}_4)_2\text{SO}_4$, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.8% KH_2PO_4 , 1% vitamin solution⁹²⁾ and 0.5% trace element solution.⁹²⁾ The accumulation of lysine in the medium increased with increased cell growth, and reached a maximum in the late stationary phase (Fig. 3). Intracellular lysine was accumulated by about 25-30 mg/g (dry wt) of cells in the early to middle logarithmic phase, and then rapidly decreased to about 10% of the highest concentration at the beginning of the stationary phase.

(5) Extracellular and Intracellular Amino Acids Accumulated by Strain SR-V-1263

Free amino acid of the culture liquid and of cells were determined at different growth stages (Table V). Lysine was predominantly found in both culture liquid and cells. In addition, arginine, glutamate and alanine were excreted when the cells were grown for 96 hr. Ornithine was also accumulated in the culture liquid incubated for 48 hr, but was not found after 96 hr. Other amino acids were not produced or only negligibly.

(6) Culture Conditions for Lysine Production by Strain SR-V-1263

Cultural conditions for lysine production were investigated by test tube scale

Lysine Biosynthesis in Yeasts

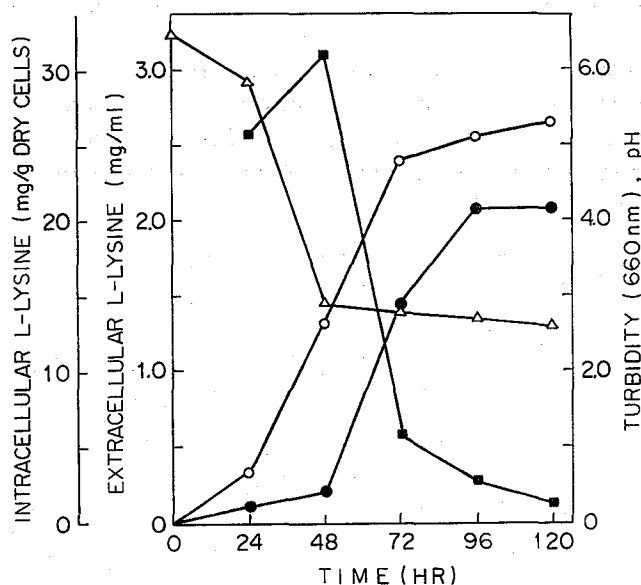


Fig. 3. Time course of lysine accumulation by strain SR-V-1263.

The mutant cells were cultured in the basal fermentation medium in a test tube with reciprocal shaking. Lysine was determined manometrically.

●, Extracellular lysine; ■, intracellular lysine; ○, cell growth; △, pH.

Table V. Extracellular and Intracellular Amino Acids Accumulated by Strain SR-V-1263

Incubation was carried out at 28°C in a test tube with reciprocal shaking. Amino acids in the culture fluid and in the cells were analyzed with a Hitachi KLA-5 automatic amino acid analyzer according to the method of Spackman *et al.*

	Extracellular (mg/ml)		Intracellular (mg/g [dry wt] of cells)	
	Cultivation time (hr)		Cultivation time (hr)	
	48	96	48	96
Ornithine	0.039	Trace	Trace	Trace
Lysine	0.115	2.24	44.1	4.02
Histidine	0	Trace	Trace	Trace
Arginine	0	0.870	Trace	2.92
Aspartate	0	Trace	Trace	Trace
Glutamate	0	0.245	15.4	0.990
Alanine	0	0.115	0.680	0.250

experiments. The mutant was grown at 28°C for 96 hr with 4 ml of fermentation medium in a test tube with reciprocal shaking.

1. *Initial pH.* The maximum productivity was observed when the mutant

were grown in a pH range of 7.0–8.0. The productivity significantly decreased at pH 9.0 (Fig. 4).

2. *Temperature.* Lysine was produced most effectively at 33°C (Fig. 4).

3. *Carbon sources.* The highest concentration of lysine was found in the basal fermentation medium containing 7.5% glucose (Fig. 4). Maltose and fructose also were the preferred carbon sources. Xylose, raffinose and galactose were only slightly assimilated or not at all.

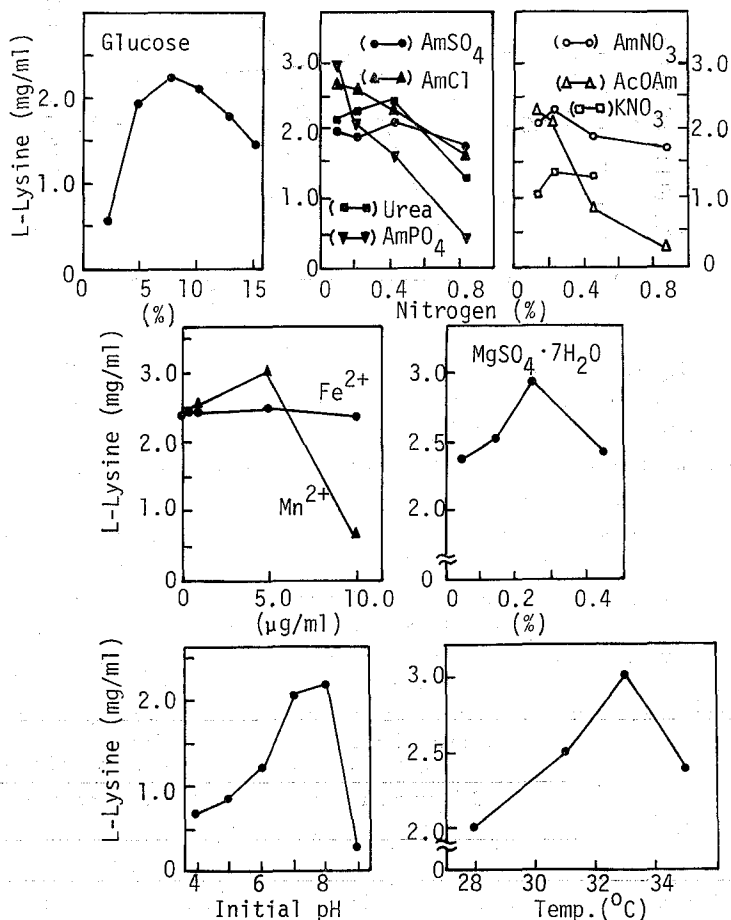


Fig. 4. Culture conditions for lysine accumulation by strain SR-V-1263.

The mutant cells were cultured in a test tube with reciprocal shaking at 28°C for 96 hr. Lysine was determined manometrically.

4. *Nitrogen sources.* When the medium containing 10% glucose was used, $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , NH_4NO_3 and urea served as good nitrogen sources (Fig. 4). A high concentration of $\text{CH}_3\text{COONH}_4$ or $(\text{NH}_4)_2\text{HPO}_4$ significantly decreased lysine accumulation.

5. *Metal ions.* Manganese ion ($5 \mu\text{g/ml}$) and magnesium ion (0.2 mg/ml) promoted lysine production by about 20%, though a high concentration of manganese

ion was rather inhibitory (Fig. 4). The addition of cobalt or nickel ions ($10\text{ }\mu\text{g/ml}$) inhibited the production by 65% and 35%, respectively, but the addition of copper or lead ions ($10\text{ }\mu\text{g/ml}$) did not affect.

6. *Effect of phosphate ions.* The concentration of lysine produced increased with increasing concentrations of KH_2PO_4 . NaH_2PO_4 was less effective, though cells were grown similarly in the presence of both phosphate salts. The optimum concentration of phosphate was 0.7% (Fig. 5). KCl , K_2SO_4 and several other potassium salts showed no influence.

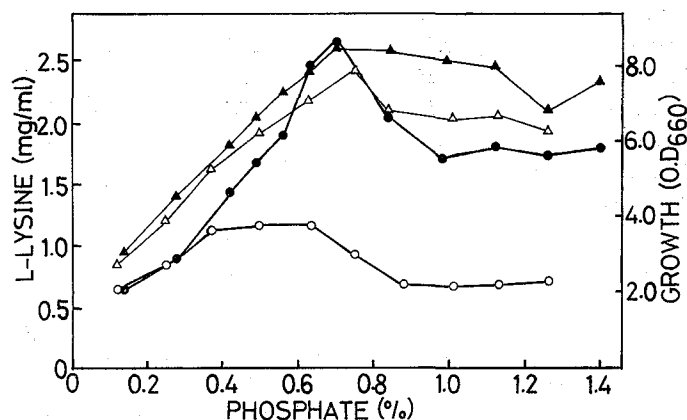


Fig. 5. Effect of KH_2PO_4 (●) and NaH_2PO_4 (○) on lysine accumulation by strain SR-V-1263.

Incubations were carried out at 28°C for 96 hr in a test tube with reciprocal shaking. Lysine was determined by a modification of the acid ninhydrin method. ▲, Cell growth (KH_2PO_4); △, cell growth (NaH_2PO_4).

Potassium ions probably more effectively stimulate the permeation of phosphate ions through the cell membrane to increase lysine production than sodium ions. Schmidt *et al.*¹⁰⁰ showed that phosphate cannot be absorbed by yeast cells in the absence of potassium ions. Gray and Bhattacharjee¹⁰¹ reported that the presence of sodium ions inhibit homocitrate synthase activity of *S. cerevisiae*. If this is the case, it is conceivable that a high concentration of potassium ions enhances lysine accumulation.

(7) Effect of Various Additions on Lysine Production by Strain SR-V-1263

Effect of various additions on lysine production was investigated in larger scale experiments: fermentation was carried out in a 300-ml Erlenmeyer flask containing 20 ml of the basal fermentation medium.

1. *Organic acids.* Organic acids tested showed remarkable stimulatory effects on both cell growth and lysine production (Table VI). The addition of 0.5% oxalate in particular enhanced lysine production three fold. Organic acids of TCA members, except oxalacetate stimulated the accumulation by 60–160%. Organic acids probably are utilized by cells as carbon sources for multiplying the yeast cells that produce lysine.

Table VI. Effect of Organic Acids on Lysine Accumulation by Strain SR-V-1263

Incubations were carried out at 28°C for 96 hr in a 300-ml Erlenmeyer flask with rotary shaking.

Organic acids	Concentration ^{a)} (%)	Growth (Turbidity at 660 nm)	Lysine ^{b)} (mg/ml)
Acetate	0.5	11.2	0.98
Citrate	0.5	11.8	1.04
α -Ketoglutarate	0.5	11.0	1.32
Succinate	0.25	9.92	1.02
Fumarate	0.25	11.7	1.51
Malate	0.25	11.6	1.26
Oxalacetate	0.5	7.94	0.73
Pyruvate	0.1	5.68	0.93
Malonate	0.25	12.7	1.56
L-(+)-Tartarate	0.5	10.4	1.23
Oxalate	0.5	9.19	1.77
DL-Lactate	0.5	8.97	0.71
Adipate	0.25	10.3	0.76
None	—	7.35	0.59

a) The effect of organic acids in the medium was tested at concentrations of 0.1, 0.25 and 0.5%. Lysine was produced in the highest yield at the indicated concentrations.

b) Lysine was determined by a modification of the acid ninhydrin method.

2. *Amino acids.* The addition of 0.5% DL- α -amino adipate enhanced lysine accumulation by about 60% (Table VII). At the concentration of 2.0%, L-glutamate, L-alanine, L-valine and glycine were also effective. L-Ornithine and L-arginine showed only slight effects at the same concentration.

3. *Organic nitrogen sources.* Organic nitrogen sources tested stimulated both cell growth and lysine production (Fig. 6). Especially, addition of 2% polypeptone promoted the productivity by about six fold. The hydrolyzates of polypeptone were analyzed and were found that 57 mg of lysine is contained per g (dry wt) of polypeptone. If the added 2% polypeptone is hydrolyzed completely by cells, 1.1 mg/ml of lysine is formed. It was ascertained that cells of wild-type strain consumed about 90% of lysine added (a final con., 1.0 mg/ml) during incubation for 96 hr. Though it was not examined the utilization of lysine by the mutant cells, it is likely that most lysine accumulated was not derived from the added polypeptone, but synthesized by cells.

4. *Detergents.* The effect of several detergents on lysine production was studied. At the concentration of 0.1%, cationic and anionic detergents in medium stimulated the productivity by about 25–45% when added after 72 hr (in the early stationary phase), but were almost ineffective when added after 48 hr (in the middle logarithmic phase) (Table VIII). For example, Table VIII shows extracellular concentration

Table VII. Effect of Amino Acids on Lysine Accumulation by Strain SR-V-1263

Incubations were carried out at 28°C for 96 hr in a 300-ml Erlenmeyer flask with rotary shaking.

Amino acids	Conc. ^{a)} (%)	Growth (Turbidity at 660 nm)	Lysine ^{b)} (mg/ml)
DL- α -Aminoadipate	0.5	8.32	0.71
L-Glutamate	0.2	10.4	0.76
L-Glutamine	0.2	9.43	0.58
L-Aspartate	0.2	9.14	0.60
L-Arginine	0.2	8.11	0.51
L-Ornithine	0.2	8.54	0.50
Glycine	0.2	9.44	0.65
L-Alanine	0.2	9.21	0.68
L-Valine	0.2	7.88	0.66
None	—	7.43	0.44

- a) The effect of amino acids in the medium was investigated at concentrations of 0.05, 0.1 and 0.2%, except for DL- α -aminoadipate. Lysine was produced in the highest yield at the indicated concentrations.
- b) Lysine was determined by a modification of the acid ninhydrin method.

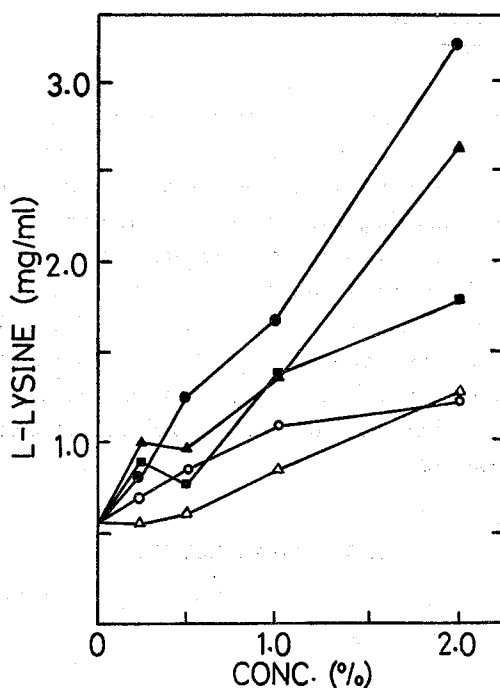


Fig. 6. Effect of organic nitrogen sources on lysine accumulation by strain SR-V-1263.

Incubations were carried out at 28°C for 96 hr in a 300-ml Erlenmeyer flask with rotary shaking. Lysine was determined by a modification of the acid ninhydrin method. ●, Polypeptone; ▲, yeast extract; ■, casaamino acid; △, corn steep liquor; ○, meat extract.

Table VIII. Effect of Addition Time of Cetyltrimethylammonium bromide (CTAB) on Lysine Accumulation by Strain SR-V-1263

Incubations were carried out at 28°C for 96 hr in a 300-ml Erlenmeyer flask with rotary shaking. At various intervals indicated, CTAB was added in the medium to a final concentration of 0.1%.

Time of addition (hr)	Growth (Turbidity at 660 nm)	Lysine ^{b)} (mg/ml)
Control ^{a)}	8.32	0.91
0	NG ^{c)}	—
12	0.314	0.15
24	0.660	0.27
36	4.64	0.57
48	6.11	0.87
60	7.02	0.92
72	9.00	1.06
84	8.69	1.21

a) Lysine was determined after 96 hr inoculation in the absence of CTAB.

b) Lysine was determined by a modification of the acid ninhydrin method.

c) No growth.

of lysine when cetyltrimethylammonium bromide (CTAB) was added at various growth stages. The addition of CTAB at the latter stages increased lysine excretion more effectively. The higher concentration of the detergents was not effective. Non-ionic detergents (Tween, Span, and Brij and Triton) had little effect.

The accompanying paper describes the mechanism of the lysine accumulation by strain SR-V-1263: the overproduction of lysine by the mutant strain is attributed to release of a feedback inhibition of homocitrate synthase by lysine, but not to a decrease in its ability of degrading intracellular lysine.⁷⁴⁾ Almost all the resistant mutants excreted lysine into the medium and they can grow well even in the presence of a high concentration of SAEC. This suggests that a regulation of homocitrate synthase of other mutants also is released.

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